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## Cow- and Quarter-Level Risk Factors for *Streptococcus uberis* and *Staphylococcus aureus* Mastitis

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### ABSTRACT

This study was designed to identify risk factors for intramammary infections with *Streptococcus uberis* and *Staphylococcus aureus* under field conditions. An 18-mo survey with sampling of all quarters of all lactating cows at 3-wk intervals was carried out in three Dutch dairy herds with medium bulk milk somatic cell count (200,000 to 300,000 cells/ml). Quarter milk samples were used for bacteriology and somatic cell counting. Data on parity, lactation stage, and bovine herpesvirus 4-serology were recorded for each animal. During the last year of the study, body condition score, and teat-end callosity scores were recorded at 3-wk intervals. A total of 93 new infections with *Strep. uberis* were detected in 22,665 observations on quarters at risk for *Strep. uberis* infection, and 100 new infections with *Staph. aureus* were detected in 22,593 observations on quarters at risk for *Staph. aureus* infection. Multivariable Poisson regression analysis with clustering at herd and cow level was used to identify risk factors for infection. Rate of infection with *Strep. uberis* was lower in first- and second-parity cows than in older cows, and depended on stage of lactation in one herd. Quarters that were infected with *Arcanobacterium pyogenes* or enterococci, quarters that had recovered from *Strep. uberis*- or *Staph. aureus*-infection in the past, and quarters that were exposed to another *Strep. uberis* infected quarter in the same cow had a higher rate of *Strep. uberis* infection. Teat-end callosity and infection with coagulase-negative staphylococci or corynebacteria were not significant as risk factors. Rate of *Staph.*

*aureus* infection was higher in bovine herpesvirus 4-seropositive cows, in right quarters, in quarters that had recovered from *Staph. aureus* or *Strep. uberis* infection, in quarters exposed to other *Staph. aureus* infected quarters in the same cow, and in quarters with extremely callused teat ends. Infection with coagulase-negative staphylococci was not significant as a risk factor. The effect of infection with corynebacteria on rate of infection with *Staph. aureus* depended on herd, stage of lactation, and teat-end roughness. Herd level prevalence of *Strep. uberis* or *Staph. aureus*, and low quarter milk somatic cell count were not associated with an increased rate of infection for *Strep. uberis* or *Staph. aureus*.

(Key words: *Streptococcus uberis*, *Staphylococcus aureus*, risk factor, mastitis)

**Abbreviation key:** BHV4 = bovine herpesvirus 4, BMSCC = bulk milk SCC, pBCS = previous BCS, PMTD = post milking teat disinfection, pSCC = previous SCC, TECR = teat-end callosity roughness, TECT = teat-end callosity thickness.

### INTRODUCTION

Mastitis is a widely occurring and costly disease in the dairy industry. The major causative agents of mastitis in modern Dutch dairy herds are *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* (Barkema et al., 1999; Miltenburg et al., 1996). In addition to the presence of bacteria, dairy herd management and cow or quarter characteristics may contribute to the occurrence of mastitis. Characteristics that increase the risk of infection can be identified in risk factor studies. The ultimate goal of such studies is to develop preventive measures to control spread of disease.

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The sampling unit in risk factor studies can be herd, cow, or udder quarter and the outcome of interest can be SCC, clinical mastitis (Peeler et al., 2000), subclinical mastitis (Busato et al., 2000), or IMI, i.e., the combination of clinical and subclinical mastitis (Lam et al., 1997; Neave et al., 1969). Some studies do not differentiate between pathogens, while others are pathogen specific and demonstrate differences between risk factors for different pathogens (Barkema et al., 1999; Schukken et al., 1991). Study designs include experimental and observational studies. The majority are cross-sectional (e.g., Peeler et al., 2000; Sischo et al., 1993), but longitudinal studies have been reported (Hogan et al., 1988; Lam et al., 1997). Cross-sectional studies deal with prevalence data. The time order of occurrence of associated factors is unknown in cross-sectional studies and causal inference is not possible (Rothman, 1986). Longitudinal studies deal with incidence data and are necessary to support a causal role of risk factors. Despite the vast body of risk factor literature, longitudinal studies are scarce because of their time-consuming nature. This is especially true for subclinical mastitis, as detection of new infections requires repeated collection and bacteriological culture of milk samples. To complicate matters, clustering of observations within cows or herds needs to be accounted for in study design or data analysis to avoid invalid statistical inference in risk factor studies (McDermott and Schukken, 1994).

Cows that have no infected quarters or multiple infected quarters occur more frequently than can be expected by chance (Barkema et al., 1997). This implies differences between cows in susceptibility to mastitis, or within cow-transmission of causative agents. Cow characteristics that influence the susceptibility to mastitis include parity, stage of lactation, and genetic make-up (Barkema et al., 1998; Busato et al., 2000; Schukken et al., 1999). Recently, bovine herpesvirus 4 (BHV4) isolation from milk was reported in association with mastitis (Wellenberg et al., 2000). No effect on susceptibility to mastitis was found for blood vitamin E level, cow conformation, retroviral infections, or BCS (Heuer et al., 1999; Schukken et al., 1999; Suriyasathaporn et al., 2000a). Quarter-level factors that affect susceptibility to mastitis include SCC and infections with minor pathogens (Hogan et al., 1988; Lam et al., 1997; Schukken et al., 1999). Most risk factors at the cow or quarter level have been identified in cross-sectional or experimental studies, or in studies on clinical mastitis.

We present a longitudinal study on the incidence of naturally occurring IMI, based on observations at quarter level at 3-wk intervals. The study was carried out in three Dutch dairy herds that implemented most measures from the five-point mastitis control plan (Hillerton et al., 1995). Risk factors for infection with *Strep.*

*uberis* and *Staph. aureus* were evaluated, while accounting for repeated measures at cow and herd level. Special attention was given to on-farm scoring of body condition and teat-end condition because clinical scores may be used as routine tools for monitoring cow and udder health in dairy herds.

## HERDS, MATERIALS, AND METHODS

### Herd Characteristics

Data were obtained from a longitudinal observational study (from May 1997 to December 1998) in three commercial dairy herds (A, B, and C) in the Netherlands. Herds were selected based on records that documented a history of at least a year of medium level bulk milk SCC (200,000 to 300,000 cells/ml) despite reasonably good udder health management (Table 1). Records were supplied by the Dutch Animal Health Service. *Staphylococcus aureus* and nonagalactiae Streptococci had been the predominant causes of infection in the herds in the preceding years. The herds were considered to be examples of a relevant level of management and bulk milk SCC (BMSCC) under current farming conditions in the Netherlands.

Herd characteristics are summarized in Table 1. Herds were housed in free-stall barns with cubicles and concrete slatted floors. Cows mostly grazed on pasture during summer (May through October), but zero-grazing was practiced in herd B during part of the summer in 1998. Animals were milked twice a day. Dry udder preparation was used in all herds. In herd A, cotton towels were used for udder preparation. Cotton towels were replaced when they looked dirty. In herds B and C, single-use paper towels were used. At every milking, the first streams of milk from each quarter were checked for signs of clinical mastitis before cluster attachment. During the study, farmers were free to make changes in herd management. Bacteriology results were reported to the farmers 3 wk after milk sample collection. In all herds, a number of cows was treated with antibiotics and/or culled because of clinical or subclinical infection with *Strep. uberis* or *Staph. aureus*. In herd B, 10 out of 14 *Strep. uberis* infected animals were housed and milked separately for 6 wk in May and June 1998. In herd C, milking clusters were flushed with hot water (90°C) after milking of *Staph. aureus* infected cows to prevent transmission of bacteria via the milking machine.

### Sampling and Data Collection

Single-quarter foremilk samples (approximately 15 ml) were collected every 3 wk from all lactating animals in each herd. Samples were taken after the first streams

**Table 1.** Characteristics of three commercial dairy herds participating in a longitudinal study on risk factors for IMI with *Streptococcus uberis* and *Staphylococcus aureus* in the Netherlands.

Characteristic	Herd A	Herd B	Herd C
Herd size (n $\pm$ SD) <sup>1</sup>	67 $\pm$ 3	95 $\pm$ 5	41 $\pm$ 2
305-d Milk production (kg) <sup>2</sup>	7187 $\pm$ 149	8166 $\pm$ 459	8508 $\pm$ 165
Bulk milk somatic cell count <sup>3</sup>	266 $\pm$ 76	235 $\pm$ 75	205 $\pm$ 69
Breed <sup>4</sup>	MRY, RH (HF, DF)	HF, DF (MRY)	HF, DF
Udder health management			
Teat dipping	no	yes/no <sup>5</sup>	yes
Yearly milk machine check-up	yes	yes	yes
Antibiotic treatment of clinical cases of mastitis	yes	yes	yes
Blanket dry cow treatment	yes	yes	yes
Routine culling of chronically infected cows	no	yes	no
Milking parlor	2 $\times$ 5 herring bone	2 $\times$ 4 open tandem	2 $\times$ 5 herring bone

<sup>1</sup>Mean number of cows present at each routine sampling during the study  $\pm$  standard deviation.

<sup>2</sup>Mean 305-d milk production (kg) during study.

<sup>3</sup>Arithmetic mean of bulk milk SCC ( $\times$  1000 cells/ml) in year preceding study.

<sup>4</sup>Dominant breed in herd, with minor breeds shown in brackets; HF = Holstein-Friesian, DF = Dutch Friesian, RH = Red Holstein, MRV = Meuse-Rhine-Yssel.

<sup>5</sup>Teat dipping was practiced from sampling 1 to 7, and from sampling 16 to 20.

of milk were discarded and after teat ends had been disinfected with cotton swabs drenched in methylated spirits (Barkema et al., 1997). At the start of the study, duplicate samples were taken on 2 consecutive days to determine the initial infection status of all lactating quarters. Additional quarter milk samples (approximately 5 ml) were collected by farmers at calving (prior to first contact with the milking machine), dry-off, culling, and in the case of clinical mastitis (any visual abnormality of milk and/or udder, with or without systemic signs of disease). In herd C, two animals were purchased during the study and sampled before entry into the milking herd. All milk samples that were used for bacteriology were stored at  $-20^{\circ}\text{C}$  until processing.

Within 3 wk of collection, 0.01 ml of milk was cultured and bacterial species were identified according to National Mastitis Council standards (Harmon et al., 1990). Colony counts were recorded for each bacterial species. A quarter was considered to have an IMI when  $\geq 1000$  cfu/ml of a pathogen (major or minor) were cultured from a single sample, when  $\geq 500$  cfu/ml of a pathogen were cultured from two out of three consecutive milk samples, when  $\geq 100$  cfu/ml were cultured from three consecutive milk samples, or when  $\geq 100$  cfu/ml were cultured from a clinical sample. Samples containing more than three bacterial species were considered contaminated and were not informative of IMI status. Samples that were culture negative during antibiotic treatment for udder disease were not considered informative of IMI status either. A previously infected quarter was considered recovered from IMI for a species if none of the above definitions were met and the sample was free of the pathogen (Zadoks et al., 2001). A fraction of the fresh quarter milk samples were preserved with sodium

azide, and used to determine SCC by a Fossomatic milk cell counter (Foss Electronic, Hillerød, Denmark) within 3 d of collection.

Data on stage of lactation, parity, clinical mastitis, and dates of calving, dry-off, or culling were available from farm records. From October 1997 (sampling 10) until the end of the study, BCS was measured for dry and lactating cows at 3-wk intervals, using a scale from 1 to 5 (where 1 = emaciated and 5 = extremely fat) with quarter intervals (Edmonson et al., 1989). At the same time, teat-end condition was scored for lactating cows, using the scale developed by Neijenhuis et al. (2000). Teat-end callosity roughness (**TECR**) was scored as smooth or rough, while teat-end callosity thickness (**TECT**) was scored as absent or thin, moderate, thick, or extreme. Scoring was done by two observers, one of whom was present at every observation.

Blood was collected from the tail vein of periparturient heifers and all lactating and dry animals once in every five samplings (at samplings 1, 6, 11, 16, 21, and 26). Serum was used to detect the presence of antibodies against BHV4 by means of an immunoperoxidase monolayer assay (Wellenberg et al., 1999). A BHV4-serostatus was assigned to each observation (milk sample) in the dataset. Serostatus was as determined on the sample date when milk and blood samples were taken on the same date. For other milk sample dates, serostatus was based on interpolation. When a change of serostatus was observed, it was assumed that the change occurred at the midpoint between two serum samplings, unless the animal went through a dry period. In that case, change of serostatus was assumed to have occurred during the dry or transitioning period (Thiry et



al., 1990) and one serostatus was assigned to each lactation.

### Herd-Level Variables

This study was not designed to identify risk factors at the herd level but rather at the cow and quarter level. However, the risk of infection may depend on herd management factors (Barkema et al., 1998; Peeler et al., 2000; Sischo et al., 1993) and on exposure to infected herd mates (Lam et al., 1996; Zadoks et al., 2001). Therefore, herd was included as an independent variable in all analyses, and the effect of prevalence of *Strep. uberis* and *Staph. aureus* on the rate of new infections was examined.

The 3-wk period between two routine samplings was called the sampling interval. For each sampling interval, prevalence of *Strep. uberis* and *Staph. aureus*, respectively, were calculated for each herd as described previously (Zadoks et al., 2001). Briefly, quarters were classified as infected or uninfected based on culture results. Duration of infection was calculated from the starting point and end point of the infected episode. The summation of number of infected quarter days in a sampling interval was considered to be the herd-level prevalence of *Strep. uberis* or *Staph. aureus* for that interval. Because an infected quarter contributes to the prevalence of infection, occurrence of a new infection can be the result or the cause of infection prevalence in that sampling interval. To allow for detection of possible cause-effect relationships, the time order of events must be known. Therefore, the prevalence of infection in the interval preceding the detection of a new infection was used as the independent variable. For example, for a new *Strep. uberis* infection detected in the fourth sampling interval, prevalence of *Strep. uberis* during the third sampling interval was taken as the value for the independent variable. Because of the definition used, the prevalence in the preceding interval was unknown for new IMI that were detected at the second sampling.

### Cow-Level Variables

Variables at the cow level included parity, the occurrence of important events (calving, clinical mastitis, dry-off, or culling), DIM, BCS, change in BCS, infection history with respect to *Strep. uberis* and *Staph. aureus*, and BHV4-serostatus. For some variables, the value concurrent with the observation of new infection was used as there could be no confusion about possible cause-effect relationships. For example, IMI does not cause parity. Parity and DIM were initially treated as categorical variables with three and six levels, respectively. For BCS, the value preceding observation of new

infection was used. Previous BCS (**pBCS**) was treated as a categorical variable with four levels (1 = 1.00 to 1.75; 2 = 2.00 to 2.75; 3 = 3.00 to 3.75; 4 = 4.00 to 5.00; Suriyasathaporn et al., 2000a). Change in BCS was calculated as the difference between the pBCS value at the current observation and the pBCS value at the previous observation, implying that it was the change in BCS over a 3-wk interval. It was treated as a categorical variable with five levels (0 = no change; 1 = 0.25 or 0.50 points increase; 2 = 0.75 or more points increase; 3 = 0.25 or 0.50 points decrease; 4 = 0.75 or more points decrease). Infection history was defined at cow level, because infection of one (or more) quarter(s) may theoretically lead to changes at systemic level, e.g., development of immunity. This could affect susceptibility to future episodes of mastitis in all quarters of the cow. Infection history in the interval preceding the observation of new IMI was used as an independent variable. A cow was considered to have a history of infection when she was infected with the pathogen of interest or when she had recovered from infection with that pathogen.

### Quarter-Level Variables

Variables defined at the quarter level were quarter position (right vs. left and front vs. rear), infection status with respect to specified major and minor pathogens, infection history with respect to *Strep. uberis* and *Staph. aureus*, SCC, TECR, TECT, and exposure to other *Strep. uberis* or *Staph. aureus*-infected quarters within the same udder. No distinction was made between exposure to one or more than one infected quarter within the udder.

For quarter position, the value concurrent with the observation of new infection was used. For other quarter-level variables the value at the observation preceding a new infection was used. In the models, previous SCC (**pSCC**) was treated as a categorical variable, because a continuous variable assumes a linear cause-effect relation. This assumption of linearity could be evaluated in the categorical response. For routine samples, five levels of quarter milk pSCC ( $\times 1000$  cells/ml) were distinguished (1 =  $<51$ ; 2 = 51 to 100; 3 = 101 to 250; 4 = 251 to 500; 5 =  $>500$ ). For samples taken by farmers (e.g., at calving or clinical mastitis), no SCC was determined. In those situations, pSCC was coded as level 6 for observations following a sample that was taken at calving or within 3 wk postcalving, and as level 7 for observations following a sample that was taken at more than 3 wk in lactation.

Infection history at quarter level was defined, in addition to the infection history at cow level, because infection of a quarter may be associated with local changes

that affect susceptibility to future episodes of mastitis in the quarter. A quarter was considered to have an infection history when it was infected with the pathogen of interest, or when it had recovered from infection with that pathogen.

Cow-level infection history was strongly correlated with quarter-level infection history and with exposure to other infected quarters within the udder. When a quarter had an infection history, or when a quarter was exposed to other infected quarters within the udder, the cow also had an infection history by definition. To be able to look at the effects of cow history (possible systemic immunity) and quarter history (possible local immunity) and within-cow exposure at the same time, we created a composite variable. Five combinations were distinguished: 1 (reference level) = no history of infection in the cow, no history of infection in the quarter, and no exposure to another infected quarter within the cow; 2 = history of infection in the cow, no history of infection in the quarter, and no exposure to another infected quarter within the cow; 3 = history of infection in the cow, no history of infection in the quarter, but exposure to another infected quarter within the cow; 4 = history of infection in the cow, history of infection in the quarter, but no exposure to another infected quarter within the cow; 5 = history of infection in the cow, history of infection in the quarter, and exposure to another infected quarter within the cow.

### Statistical Analyses

Occurrence or nonoccurrence of new infection was the dependent variable in all analyses. Separate analyses were run for new infection with *Strep. uberis* and *Staph. aureus*, respectively. Observations from quarters with existing infections of the pathogen under analysis were excluded from the dataset. Infection status before parturition was unknown in heifers and management of dry cows was different from management of lactating cows. Therefore, infections that were first detected at calving were also excluded from analysis.

First, univariate analysis of all data was performed to detect extreme values. No observations were excluded for this reason. Next, bivariate analysis for screening of independent variables was done in a Poisson regression model that always included herd as a fixed effect. Mastitis incidence is a person-time rate (number of disease onsets per sum of time at risk for all population members) and use of Poisson regression is conventional for analysis of person-time rates (Greenland, 1998). Finally, all independent variables with  $P \leq 0.20$  for at least one level in the bivariate analysis were submitted to a multivariate mixed model, based on general estimation equations (Zeger et al., 1988).

Two-way interactions between herd, parity, DIM, quarter position, pSCC, previous infection status with respect to CNS, previous infection status with respect to corynebacteria, BHV4-serostatus, TECR, and TECT were also tested for statistical significance in the multivariable model, with the exception of interaction between serostatus and quarter position. The latter interaction was not considered biologically meaningful.

In the dataset, clustering of observations occurred at multiple levels. Repeated observations over time were clustered within quarters, quarters were spatially clustered within cows, and cows were clustered within herd. Modeling of multiple levels of clustering is possible using the GLIMMIX Macro (Little et al., 1996). Due to the small number of new infections relative to the total number of observations in the dataset, computational limitations arose with this method (nonconvergence). Using the VARCOMP procedure (SAS/STAT, 1990) in SAS version 8.1, we determined that cow accounted for more variability than quarter. Therefore, analyses were run with herd as fixed effect and cow as repeated effect, accounting for correlation at herd level and cow level, but not at quarter level. Compound symmetry was used as the covariance structure for within-cow correlation (Barkema et al., 1997). Analyses were run with the GENMOD procedure of SAS version 8.1, using a regression model with log link and Poisson distributed error. A forward stepwise analysis of main effects and interactions was performed with cut-off for retention set at  $P \leq 0.10$  in the likelihood ratio test. The linearized mixed model was as follows:

$$\log P(Y = y) = \beta_0 + \sum \beta_i RF_i + R + \varepsilon, \quad [1]$$

where Y is a random variable measuring the number of new infections, y is the actual realization of Y,  $\beta_0$  = intercept,  $\beta_i$  = regression coefficient for risk factor i,  $RF_i$  = value or level of risk factor i, R = random cow effect, and  $\varepsilon$  = residual error. Regression coefficients ( $\beta_i$ ) are the natural logarithm of the rate ratio. The rate ratio is the rate of new infections in quarters at a specified level of a risk factor, relative to the rate of new infections in quarters at the reference level for that risk factor.

Model fit of the final model was evaluated using the ratio of the Pearson chi-square statistic to the remaining degrees of freedom (McDermott et al., 1994). There is no standard method to assess the amount of variability in the dependent variable that is accounted for by the independent variables in a Poisson regression model. Therefore, the ability of the model to differentiate between occurrence and nonoccurrence of new infection was assessed semiquantitatively. Predicted values for the probability of new infection were calculated in

the GENMOD procedure of SAS (SAS/STAT, 1990), and compared between observations of nonoccurrence and occurrence of new infection, respectively. Sensitivity and specificity of the model as a test for prediction of new infection were calculated after selection of an appropriate cut-off value.

For one independent variable, the proportion of new infections in all observations that could be attributed to the exposure was calculated using equation [2] (Rothman, 1986):

$$AP_T = \frac{RR - 1}{RR + 1/P_0 - 1} \quad [2]$$

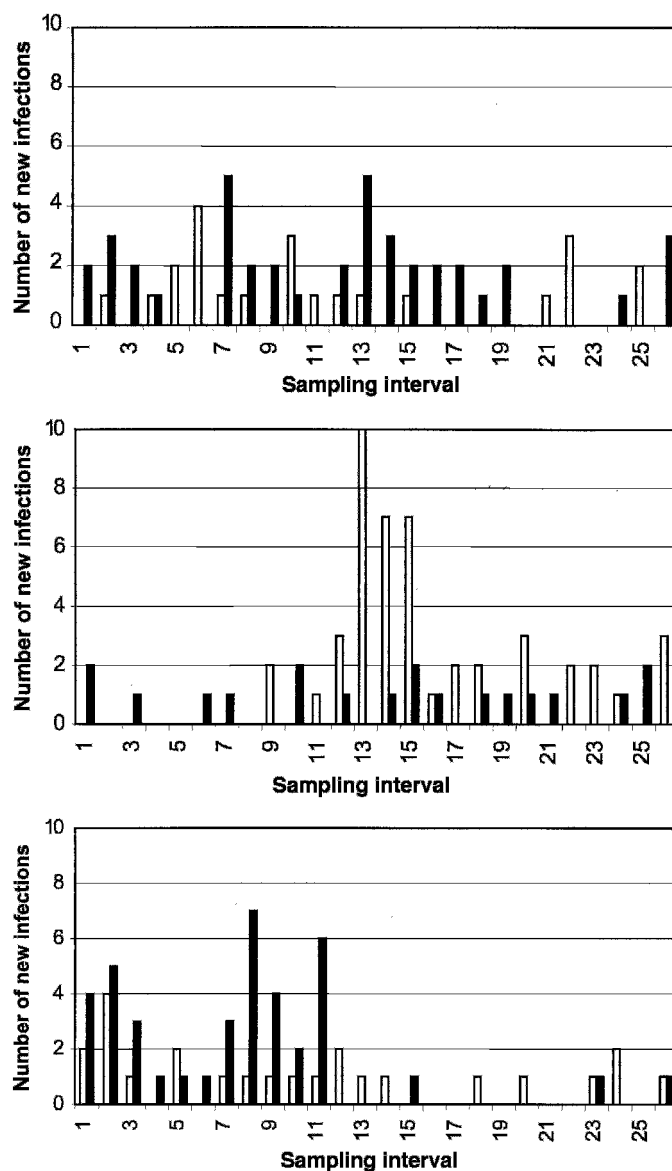
with:  $AP_T$  = attributable proportion in all observations.  $RR$  = rate ratio (incidence rate in exposed observations/incidence rate in unexposed observations),  $P_0$  = proportion of all observations that is exposed.

The datasets for quarters at risk of *Strep. uberis* mastitis and *Staph. aureus* mastitis were used to create multiple subsets. The first dataset for either pathogen (FULLset) contained data on herd, important events (clinical mastitis, dry-off, culling), parity, DIM, quarter position, previous infection status with respect to major and minor pathogens, pSCC, previous recovery history at cow and quarter level, and previous exposure to other infected quarters. The second dataset (PREVset) was a subset of FULLset and contained the same variables plus data on the herd level prevalence of *Strep. uberis* or *Staph. aureus* in the previous sampling interval. The third dataset (SEROset) was a subset of FULLset that included BHV4-serostatus and excluded observations that had missing values for BHV4-serostatus. The last dataset (SCOREset) was a subset of FULLset that contained observations with values for pBCS and previous TECR and TECT. Data(sub)sets for analysis of risk factors for *Strep. uberis* mastitis will be referred to as StrepFULLset, StrepPREVset, StrepSEROset, and StrepSCOREset. Data(sub)sets for analysis of risk factors for *Staph. aureus* mastitis will be referred to as StaphFULLset, StaphPREVset, StaphSEROset, and StaphSCOREset.

## RESULTS

### Descriptive Results

During the 81-wk study period, 93 new infections with *Strep. uberis* and 100 new infections with *Staph. aureus* were detected. Figure 1a through 1c shows the number of new infections per 3-wk sampling interval for each herd. The number of observations on quarters at risk for *Strep. uberis* and *Staph. aureus* infection, respectively, and the total number of new infections are



**Figure 1a.** Number of new infections with *Streptococcus uberis* (white bars) and *Staphylococcus aureus* (black bars) per 3-wk sampling interval in herd A. Size of the lactating herd ( $n \pm SD$ ) was  $67 \pm 3$  cows. **b)** Number of new infections with *Strep. uberis* (white bars) and *Staph. aureus* (black bars) per 3-wk sampling interval in herd B. Size of the lactating herd ( $n \pm SD$ ) was  $95 \pm 5$  cows. **c)** Number of new infections with *Strep. uberis* (white bars) and *Staph. aureus* (black bars) per 3-wk sampling interval in herd C. Size of the lactating herd ( $n \pm SD$ ) was  $41 \pm 2$  cows.

summarized per herd in Table 2. The infections with *Strep. uberis* were detected in 81 quarters of 56 cows. The infections with *Staph. aureus* were detected in 91 quarters of 66 cows. The maximum number of infections per cow and quarter was five and three, respectively, for *Strep. uberis* and six and three for *Staph. aureus*.

**Table 2.** Number of observations per herd and number of new infections per herd included in analysis of risk factors for *Streptococcus uberis* and *Staphylococcus aureus* infection.

Herd	<i>Streptococcus uberis</i>				<i>Staphylococcus aureus</i>			
	Observations		New infections		Observations		New infections	
	n	%	n <sup>1</sup>	%	n	%	n <sup>2</sup>	%
A	7454	32.9	23	24.7	7256	32.1	41	41.0
B	10,625	46.9	46	49.5	10,752	47.6	19	19.0
C	4586	20.2	24	25.8	48,585	20.3	40	40.0
Total	22,665	100.0	93	100.0	22,593	100.0	100	100.0

<sup>1</sup>Infections were observed in 81 quarters of 56 cows.

<sup>2</sup>Infections were observed in 91 quarters of 66 cows.

### Risk Factors for *Streptococcus uberis* Infection

An overview of independent variables and their significance in bivariate and multivariable analysis of risk factors for new infection with *Strep. uberis* is given in Table 3. Of all interactions that were tested, only the interaction between herd and DIM was significant. Parameter estimates for significant variables and interactions in the multivariable model are listed in Table 4. Clinical signs in the quarter were significantly ( $P < 0.0001$ ) associated with new *Strep. uberis* infection, but this variable was not included in the multivariable models. The time order of occurrence of clinical signs and new infection is unknown and therefore causal inference was not possible.

The number of levels was reduced for several categorical variables. For DIM, there was no difference between estimates for 181 to 240, 241 to 320, or more than 320 DIM, and the levels were collapsed into the reference level. When herd and DIM were included in the model, but the interaction between herd and DIM was not, the remaining levels of DIM were all significantly associated with an increased risk of *Strep. uberis* mastitis. For 1 to 60 DIM, the relative risk was 2.8 ( $P < 0.001$ ), for 61 to 120 DIM the relative risk was 1.9 ( $P = 0.10$ ), and for 121 to 180 DIM the relative risk was 2.0 ( $P < 0.05$ ). When the interaction between herd and DIM was added to the model, overall model fit improved, as indicated by the likelihood ratio test. The level of 61 to 120 DIM in herd 2 remained as the only level with a significantly increased risk of new infection compared with the reference level (>180 DIM in herd 3), with a relative risk of 7.5 ( $P < 0.05$ ).

For quarters that had not recovered from *Strep. uberis* infection in the past, and that were not currently exposed to infected quarters within the same cow, there was no effect of cow-level recovery from *Strep. uberis* infection. The composite variable for cow- and quarter-level infection history and exposure could thus be reduced to two binary variables, i.e., quarter-level infection history and exposure within the udder. Both were

significant as independent variables, while their interaction was not significant.

For prediction of *Strep. uberis* infections, the association between pSCC level and new infection was nonlinear. Therefore, pSCC could not be treated as a continuous independent variable. In bivariate analysis, level 5 (> 250,000 cells/ml) and 7 (preceding value missing at more than 3 wk in lactation) of pSCC were significantly associated with increased risk of new infection but pSCC was not significant in the multivariable model.

StrepPREVset, StrepSEROset, and StrepSCOREset contained 21,791, 22,077, and 14,493 observations, and 85, 87, and 56 new *Strep. uberis* infections, respectively. None of the variables that could only be tested in StrepPREVset, StrepSEROset, or StrepSCOREset were significant.

Model sensitivity (ability to predict occurrence of new infection) was over 50% and model specificity (ability to predict nonoccurrence of new infection) was over 90% for the model based on StrepFULLset. The Pearson chi-square statistic was 0.94 per degree of freedom, indicating good model fit.

The effect of preceding infection with enterococci on other parameter estimates was evaluated, because it was thought that diagnostic interpretation played a role for this variable (see Discussion). When preceding infection with enterococci was omitted from the model for StrepFULLset, estimates for other independent variables changed little in direction, order of magnitude, or significance of effect. The proportion of *Strep. uberis* infections that was attributable to exposure to preceding infection with enterococci, i.e., the attributable proportion (AP<sub>T</sub>), was 5.1%.

### Risk Factors for *Staphylococcus aureus* Infection

Independent variables and their significance in bivariate and multivariable analysis of risk factors for new infection with *Staph. aureus* are listed in Table 3. Several two-way interactions were significant as predictors for new infection. Parameter estimates for significant



**Table 3.** Significance of independent variables in bivariate and multivariable Poisson regression models for prediction of new infections with *Streptococcus uberis* or *Staphylococcus aureus*, respectively. + = significant ( $P \leq 0.10$ ); – = nonsignificant; . . . = no estimate (no new infections); x = not tested.

Independent variable	<i>Strep. uberis</i> model		<i>Staph. aureus</i> model	
	Bivariate	Multivariable	Bivariate	Multivariable
Herd level variables				
Herd of origin	–	–	+	+
<i>Strep. uberis</i> prevalence	–	–	x	x
<i>Staph. aureus</i> prevalence	x	x	–	–
Cow level variables				
Extra samples				
Clinical mastitis <sup>1</sup>	+	x	+	x
Dry-off	–	–	–	–
Culling	–	–	. . .	. . .
Parity	+	+	+	+
DIM	+	+	+	+
Recovery from <i>Strep. uberis</i>	–	–	–	–
Recovery from <i>Staph. aureus</i>	–	–	–	–
Bovine herpesvirus 4-serostatus	–	–	+	+
Previous BCS	–	–	–	–
Change in previous BCS <sup>2</sup>	–	–	–	–
Quarter level variables				
Right vs. left	–	–	+	+
Front vs. rear	–	–	+	–
Recovery from <i>Strep. uberis</i>	+	+	+	+
Recovery from <i>Strep. dysgalactiae</i>	+	–	–	–
Recovery from <i>Staph. aureus</i>	+	+	+	+
Previous infections status				
<i>Arcanobacterium pyogenes</i>	+	+	. . .	. . .
<i>Bacillus</i> species	–	–	–	–
Coagulase-negative staphylococci	–	–	–	–
Corynebacteria	–	–	–	+
Enterobacteria	+	+	–	–
<i>Escherichia coli</i>	–	–	–	–
<i>Staph. aureus</i>	–	–	x	x
<i>Strep. dysgalactiae</i>	+	–	+	–
<i>Strep. uberis</i>	x	x	–	–
Other streptococcus species <sup>3</sup>	+	+	–	–
Exposure within udder to quarters with				
<i>Staph. aureus</i>	x	x	+	+
<i>Strep. uberis</i>	+	+	x	x
Previous quarter SCC	+	–	+	+
Preceding teat end callosity				
Roughness	–	–	–	+
Thickness	–	–	+	+

<sup>1</sup>Significant in bivariate and multivariable models for *Strep. uberis* and *Staph. aureus* ( $P < 0.0001$ ) but excluded from multivariable analysis because of unknown order of occurrence of new infection and clinical signs.

<sup>2</sup>Estimates for change in previous BCS based on 12,187 observations in the *Strep. uberis* dataset, and on 12,207 observations in the *Staph. aureus* dataset.

<sup>3</sup>*Streptococcus agalactiae* was never isolated during the study.

independent variables and interactions for *Staph. aureus* are listed in Table 4, with the exception of the interaction between parity and front or rear quarter. The interaction between parity and front or rear quarter was significant according to the likelihood ratio test. There was no difference between front or rear quarters for parity 2 ( $P = 0.36$  in Wald's test) or higher ( $P = 0.88$  in Wald's test), but front quarters were significantly associated with a higher rate of infection in first-parity animals (17 new infections in front quarters and three new infections in hind quarters;  $P < 0.01$ ). Of the inter-

actions terms that were statistically significant in the model for *Staph. aureus*, interaction between parity and quarter position seemed least likely to be biologically meaningful. Addition of this interaction to the model did not change significance, or size or direction of effect for other parameter estimates (results not shown). We decided to omit the interaction between parity and quarter position from the model to facilitate biological interpretation of other interactions that were maintained in the model. Clinical signs in the quarter were significantly associated with new *Staph. aureus* infec-

**Table 4.** Parameter estimates with standard error from multivariable Poisson regression models for prediction of new infections with *Streptococcus uberis* or *Staphylococcus aureus* during lactation. Variables or interactions were significant ( $P \leq 0.10$ ) according to likelihood ratio test. Superscripts indicate significance of levels of categorical variables according to Wald's test.

Independent variable	StrepFULLset <sup>1</sup> n <sub>obs</sub> = 22,665 <sup>2</sup> n <sub>new</sub> = 93 <sup>2</sup>	StaphFULLset n <sub>obs</sub> = 22,593 n <sub>new</sub> = 100	StaphSEROset n <sub>obs</sub> = 22,015 n <sub>new</sub> = 94	StaphSCOREset n <sub>obs</sub> = 14,386 n <sub>new</sub> = 53
Intercept	-5.68 ± 0.40** <sup>3</sup>	-5.27 ± 0.37**	-5.55 ± 0.44**	-5.47 ± 0.55**
Herd				
A	-0.66 ± 0.53 <sup>NS</sup>	0.06 ± 0.32 <sup>NS</sup>	-0.44 ± 0.40 <sup>NS</sup>	-0.05 ± 0.40 <sup>NS</sup>
B	-0.66 ± 0.48 <sup>NS</sup>	-0.62 ± 0.34†	-1.25 ± 0.34**	-1.04 ± 0.43*
C	Reference	Reference	Reference	Reference
Parity				
First	-0.55 ± 0.33†	-0.43 ± 0.27 <sup>NS</sup>	-0.33 ± 0.29 <sup>NS</sup>	-1.37 ± 0.44**
Second	-0.51 ± 0.30†	-0.75 ± 0.29**	-0.64 ± 0.30*	-1.57 ± 0.52**
Third and higher	Reference	Reference	Reference	Reference
DIM				
1 to 60 d	0.52 ± 0.82 <sup>NS</sup>	-0.60 ± 0.50 <sup>NS</sup>	-0.46 ± 0.53 <sup>NS</sup>	-0.12 ± 0.68 <sup>NS</sup>
61 to 120 d	-0.52 ± 0.77 <sup>NS</sup>	-0.04 ± 0.41 <sup>NS</sup>	0.05 ± 0.43 <sup>NS</sup>	0.79 ± 0.45†
121 to 180 d	0.14 ± 0.51 <sup>NS</sup>	0.30 ± 0.46 <sup>NS</sup>	0.36 ± 0.48 <sup>NS</sup>	0.22 ± 0.47 <sup>NS</sup>
> 180 d	Reference	Reference	Reference	Reference
BHV4-seropositive	n.a. <sup>4</sup>	n.a.	0.79 ± 0.34*	NS
Right quarter	NS	0.38 ± 0.19*	0.32 ± 0.19†	NS
Quarter recovered from				
<i>Strep. uberis</i> IMI	1.10 ± 0.38**	0.85 ± 0.36*	0.99 ± 0.36**	1.22 ± 0.42**
<i>Staph. aureus</i> IMI	0.72 ± 0.36*	0.88 ± 0.32**	0.72 ± 0.35*	NS
Preceded by IMI with				
<i>Arcanobacterium</i>	3.06 ± 1.02**	NS	NS	n. e.
Enterococci	2.01 ± 0.39**	NS	NS	n. e.
Corynebacteria	NS	0.26 ± 0.43 <sup>NS</sup>	0.27 ± 0.44 <sup>NS</sup>	-2.38 ± 0.94**
Exposure within udder to				
<i>Strep. uberis</i>	1.44 ± 0.27**	n.a.	n.a.	n.a.
<i>Staph. aureus</i>	n.a.	1.53 ± 0.31**	1.47 ± 0.32**	1.01 ± 0.43*
Previous SCC	NS			
< 251,000 cells/ml		Reference	Reference	Reference
251,000 to 500,000		0.74 ± 0.36*	0.59 ± 0.40 <sup>NS</sup>	0.53 ± 0.50 <sup>NS</sup>
> 500,000		1.06 ± 0.28**	1.05 ± 0.30**	1.12 ± 0.40**
Missing < 21 DIM		1.36 ± 0.57*	1.41 ± 0.61*	n. e.
Missing > 20 DIM		-0.24 ± 0.55 <sup>NS</sup>	-0.14 ± 0.56 <sup>NS</sup>	n. e.
DIM * herd		NS	NS	NS
1 to 60 d, herd A	0.80 ± 0.98 <sup>NS</sup>			
61 to 120 d, herd A	0.15 ± 1.06 <sup>NS</sup>			
121 to 180 d, herd A	0.08 ± 0.80 <sup>NS</sup>			
1 to 60 d, herd B	0.55 ± 0.96 <sup>NS</sup>			
61 to 120 d, herd B	2.01 ± 0.91**			
121 to 180 d, herd B	1.03 ± 0.65 <sup>NS</sup>			
Corynebacteria <sup>5</sup> * herd	NS			NS
Present, herd A		-1.10 ± 0.52*	-1.09 ± 0.55*	
Present, herd B		-1.63 ± 0.66*	-1.47 ± 0.69*	
Corynebacteria <sup>5</sup> * DIM	NS			NS
Present, 1 to 60 d		0.48 ± 0.82 <sup>NS</sup>	-0.22 ± 1.09 <sup>NS</sup>	
Present, 61 to 120 d		1.24 ± 0.55*	1.24 ± 0.56*	
Present, 121 to 180 d		-0.19 ± 0.71 <sup>NS</sup>	-0.04 ± 0.73 <sup>NS</sup>	
Teat-end callosity	n.a.	n.a.	n.a.	
Rough, corynebacteria absent <sup>5</sup>				0.25 ± 0.46 <sup>NS</sup>
Rough, corynebacteria present <sup>5</sup>				2.25 ± 0.99*
Thickness				
Absent/thin				reference
Moderate/thick				0.24 ± 0.37 <sup>NS</sup>
Extreme				1.62 ± 0.44**

<sup>1</sup>StrepFULLset = Full dataset on quarters at risk of *Strep. uberis* infection; StaphFULLset = full dataset on quarters at risk of *Staph. aureus* infection; StaphSEROset = subset of StaphFULLset including BHV4-serostatus; StaphSCOREset = subset of StaphFULLset including BCS and teat-end callosity scores.

<sup>2</sup>n<sub>obs</sub> = Number of observations; n<sub>new</sub> = number of new infections.

<sup>3</sup>NS = Not significant (superscript indicates nonsignificant level of categorical variable; normal script indicates nonsignificant variable); † $P \leq 0.10$ ; \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ .

<sup>4</sup>n.a. = Not applicable; n. e. = no estimate (model did not converge).

<sup>5</sup>Refers to presence or absence of corynebacteria at preceding sampling.

tion ( $P < 0.0001$ ), but were not included in the multivariable models because the cause-effect order of the associated variables was unknown.

As before, the number of levels was reduced for several categorical variables. For DIM there was no difference between estimates for 181 to 240, 241 to 320, and more than 320 DIM, and the levels were combined into the reference level. The association between pSCC level and new infection was more or less linear for *Staph. aureus* and a higher pSCC level was associated with a higher risk of new infection compared with the original reference level ( $<50,000$  cells/ml). The differences between estimates for  $<50,000$ , 51,000 to 100,000, and 101,000 to 250,000 cells/ml were not significant, however, and the three levels were combined into a new reference level. The variable was kept in the model as a categorical variable to facilitate interpretation of parameter estimates, including estimates for observations with missing values. Cow-level history of *Staph. aureus* infection was not significant. As for *Strep. uberis*, the composite variable for cow and quarter-level history and exposure was reduced to its significant constituent parts, i.e., quarter-level history of infection and within-cow exposure.

StaphPREVset, StaphSEROset, and StaphSCOREset contained 21,729, 22,015, and 14,386 observations and 92, 94, and 53 new *Staph. aureus* infections, respectively. *Staph. aureus* prevalence was not significant as an independent variable, but BHV4-serostatus was significant in the *Staph. aureus* model. The proportion of BHV4-seropositive observations was 84.1% for herd A, 78.6% for herd B, and 13.8% for herd C. TECT and TECR were both significant as predictors of new *Staph. aureus* infection in the multivariable model (Table 4).

Model sensitivity was around 50% and model specificity was around 90% for all models. The Pearson chi-square statistics per degree of freedom were 1.02, 0.96, and 0.83, respectively, for models based on StaphFULset, StaphSEROset, and StaphSCOREset.

## DISCUSSION

### Herd Level

Longitudinal studies with repeated measurements of IMI status are necessary to identify factors associated with a change of infection status, but they are expensive and rare. The study presented here was specifically designed to identify risk factors for new infections with *Strep. uberis* and *Staph. aureus* under field conditions. During the study, the farmers received reports on the infection status of their animals every 3 wk. Using that information and elements from the five-point mastitis control plan, all farmers managed to control the incidence of *Staph. aureus* mastitis in their herds (Figure

1). Similar reduction in incidence of *Staph. aureus* mastitis has been observed by others (Hillerton et al., 1995; Neave et al., 1969). Control of *Strep. uberis*, e.g., through disinfection of milking clusters or treatment or culling of infected cows, was less strict in the study herds and an outbreak of *Strep. uberis* mastitis occurred in one herd. Management factors that may have contributed to this outbreak have been described elsewhere (Zadoks et al., 2001).

Infected mammary glands are considered to be the main source of *Staph. aureus* and one of several sources of *Strep. uberis* in dairy herds (Leigh, 1999; Neave et al., 1969). In previous studies on *Staph. aureus* mastitis and *Strep. uberis* mastitis, prevalence of pathogens was a significant predictor for the number of new infections (Lam et al., 1996; Zadoks et al., 2001). In the current study, herd level prevalence of pathogens was not significant as a risk factor for new infection. This difference can most likely be attributed to the conscious preventive action taken in the herds involved in the current study or to the limited number of herds under observation. In addition, the dynamics of infection could differ between outbreaks or epidemic situations when most of the herd is susceptible and has not been exposed to the pathogen, and steady states or endemic situations in which most of the population has been in contact with the pathogen. This would be in agreement with the results described by Lam (1996) and Zadoks (2001) and their co-workers, in which transmission differed between outbreak and postoutbreak phases of the studies. In the data presented here, herd level prevalence of *Strep. uberis* was significant as an independent variable for a subset of the data that covered the outbreak in herd B (results not shown).

Observations on cows or quarters within a herd are not independent. The within-herd correlation or dependency of measures must be considered in study design or data analysis to avoid invalid statistical inference (Barkema et al., 1997; McDermott and Schukken, 1994). Within-herd correlation is likely to differ between herds, as management differs between herds. When a common within-herd correlation cannot be assumed, mixed models with fixed effects for herd and additional random effects for subgroups within herds, e.g., cows, are needed (McDermott et al., 1994). In our study, within-herd correlation was not the subject of interest, but rather an effect that had to be corrected for to estimate the effect of cow and quarter-level variables correctly. This correction was done as suggested by McDermott et al. (1994). In addition, interactions between herd and other main effects were tested for significance to detect cow or quarter-level risk factors that were significant in some herds, but not in others.

### Cow Level

Clustering of observations also occurred at cow and quarter level. When correlation within cow (spatial clustering) and correlation within quarter (temporal clustering) were both modeled, computational limitations arose. Difficulties in fitting of multilevel models with statistical software have been recognized before (Barkema et al., 1997). We chose to model within-cow correlation, because cow explained more variance than quarter when specified risk factors were not included in the model. Compound symmetry in which the covariance matrix has constant variance and constant correlation was chosen to model within-cow correlation, as had been done by others (Barkema et al., 1997; Schukken et al., 1999). Alternatives such as autoregressive or unstructured covariance matrices seemed less justified biologically. Due to the high number of observations per cow (up 108), they would accentuate differences rather than similarities between repeated observations. By treating all observations within a cow as being equally correlated, spatial clustering (quarters within cow) and temporal clustering (consecutive samples within quarters) were treated as if they were the same. Given the limitations of the dataset, this seemed the best way to model the biology of the repeated measures. Clustering of data commonly causes overdispersion, resulting in a value higher than one for the Pearson chi-square statistic per degree of freedom (McDermott et al., 1994). In our models, this statistic had a value close to one, indicating good model fit and suggesting appropriate model selection.

Significant independent variables at cow level included parity and DIM. Incidence of *Strep. uberis* and *Staph. aureus* IMI was lower in first- and second-parity cows than in third- or higher-parity cows. This is in line with results for incidence of clinical mastitis (Barkema et al., 1998; Miltenburg et al., 1996) and prevalence of subclinical mastitis (Busato et al., 2000). Increased prevalence may be the result of increased incidence or increased duration of infection. We showed that increased prevalence is at least partly due to higher incidence in multiparous cows. For *Staph. aureus*, the association between DIM and rate of infection was affected by infection with corynebacteria. Infection with corynebacteria was associated with increased risk of infection at peak lactation (61 to 120 DIM). For *Strep. uberis*, the association between DIM and rate of new infection differed with herd. Peak lactation (61 to 120 DIM) was associated with increased rate of infection in one herd. Several studies document a high incidence of clinical mastitis in early stages of lactation (Barkema et al., 1998; Miltenburg et al., 1996), but we have not found any reports on incidence

of IMI (i.e., clinical as well as subclinical mastitis) in relation to stage of lactation to which we can compare our results.

Increased incidence of mastitis during early or peak lactation may be a result of negative energy balance (Suriyasathaporn et al., 2000b). In dairy health management, change in BCS is used as an indicator of energy balance. In our study, we found no effect of BCS or change in BCS on rate of new infections with *Strep. uberis* or *Staph. aureus*. Similar results have been obtained for increase of SCC (Suriyasathaporn et al., 2000a) and for occurrence of clinical mastitis (Heuer et al., 1999). The failure of the current and other studies to demonstrate significance of BCS as a risk factor for mastitis in multivariable analyses may be due to differences in definitions of mastitis and energy balance, to lack of power in each study, or to inadequacy of body condition scoring as a tool to measure energy balance under field conditions.

BHV4-seroprevalence in study herds A (84% of milk samples from seropositive animals) and B (79% of milk samples from seropositive animals) was high compared with seroprevalence in Dutch cattle in general (18%; Wellenberg et al., 1999). BHV4 is a worldwide distributed virus of cattle that is not associated with clearly defined clinical entities (Thiry et al., 1990). The virus has been isolated from milk of cows with clinical mastitis that also harbored *Strep. uberis* or *Escherichia coli*. Seroconversion to BHV4 did not differ significantly between case cows and matched controls (Wellenberg et al., 2000). In our study, BHV4-serostatus was a significant risk factor for *Staph. aureus* infection. BHV4-positive animals had a higher rate of infection than BHV4-negative animals. The observed statistical association could be a chance effect, the result of increased susceptibility to infections in certain animals (resulting in IMI and in BHV4-infection), or the result of increased susceptibility to mastitis caused by latent BHV4-infection. In latent infections, BHV4 is predominantly situated in nervous ganglia and in mononuclear blood cells. Persistent infection of mononuclear cells with BHV4 may reduce their phagocytic functions (Thiry et al., 1990). Theoretically, the phagocytic capacity of udder monocytes and macrophages could be reduced by BHV4-infection, which could explain the increased susceptibility to IMI. However, polymorphonuclear cells are thought to be more important than mononuclear cells in the protection of the udder against *Staph. aureus* infection (Nickerson and Heald, 1982). By contrast, mononuclear cells are considered to be more important than polymorphonuclear cells in *Strep. uberis* IMI (Leigh, 1999), but BHV4-serostatus was not significant as a risk factor in the *Strep. uberis* model. Further study into the role of BHV4 in bovine mastitis seems warranted.



## Quarter Level

Quarter position has been described as a risk factor in studies on incidence of clinical mastitis and prevalence of subclinical mastitis (Barkema et al., 1997; Busato et al., 2000; Miltenburg et al., 1996). The IMI was found more often in rear quarters than in front quarters. In our study, front versus rear quarter was significant in univariate analysis for *Staph. aureus*, but not in multivariable analysis. Studies by Barkema (1997) and Miltenburg (1996) and their co-workers only describe univariate analysis. Busato et al. (2000) found more mastitis in rear quarters in late lactation, based on multivariable analysis. Because their study, though presented as a longitudinal study, is in fact a repeated cross-sectional study, it is not possible to distinguish between incidence and duration of infection as the cause of higher prevalence in rear quarters. Right quarters had a higher rate of *Staph. aureus* infection than left quarters in our study. A higher prevalence of infection in right quarters was also found by Barkema et al. (1997) and could be associated with lying behavior of cows (Ewbank, 1966). For a limited number of herds as in our study, transmission of *Staph. aureus* via teat cup liners (O'Shea, 1987) may also explain a higher rate in specific quarters, e.g., right quarters.

Quarters that had recovered from *Strep. uberis* or *Staph. aureus* mastitis had a higher rate of infection than quarters that had not experienced infection before. This means that recovery from infection does not confer immunity to reinfection with the same pathogen. Recovery from infection was a risk factor for reinfection with the homologous bacterial species, but also for infection with the heterologous bacterial species. It seems that some quarters are more susceptible to infection than others, irrespective of pathogen. Our field observations were in contrast to reports of immunity to reinfection with *Strep. uberis* (Hill, 1988). In Hill's experimental study, the first line of defense was circumvented.

For *Strep. uberis*, preceding infections with enterococci were associated with an increased rate of new infection. No other minor pathogens were associated with increased rate of *Strep. uberis* infection. It is known that identification of streptococci and enterococci based on growth and biochemical characteristics is difficult (Leigh, 1999). Therefore, we think that enterococci and streptococci may have been misclassified in some cases. Because the attributable proportion of new *Strep. uberis* was low for infection with enterococci, and because other parameter estimates were not affected much by the addition or removal of this risk factor, we consider results from the *Strep. uberis* model to be valid despite possible occurrence of misclassification. We recommend that in studies of *Strep. uberis* mastitis all

enterococcal and streptococcal isolates be tested with more sensitive and specific techniques than standard NMC-recommended methods alone.

Preceding infection with coagulase-negative staphylococci or corynebacteria was not associated with an increased or decreased rate of *Strep. uberis* infection. Hogan et al. (1988) found an increased rate of infections with environmental streptococci in quarters infected with *Corynebacterium bovis* or *Staphylococcus* species. In their study, within-cow correlation and quarter-level risk factors were not taken into account, and no distinction was made between *Strep. uberis* and *Strep. dysgalactiae*. Lam et al. (1997) found a protective effect of corynebacteria but not CNS on subsequent infection with *Strep. uberis*. By performing a within-cow comparison of case and control quarters, cow effects were corrected for. Quarter characteristics such as pSCC or teat-end condition were not accounted for in their study. For *Staph. aureus* infection, CNS were not significant as a risk factor in our study, in agreement with reports by other authors (Lam et al., 1997; Schukken et al., 1999). The situation for corynebacteria is more complex. Corynebacteria were not significant as a risk factor for *Staph. aureus* IMI in late-lactation animals in herd C (reference level). However, infection with corynebacteria was associated with a lower rate of *Staph. aureus* IMI in herds A and B, and with a higher rate of *Staph. aureus* IMI at peak lactation (61 to 120 d) and in rough teat ends. Schukken et al. (1999) found a protective effect of corynebacteria after experimental infections. They used intracisternal challenge with *Staph. aureus*, thus surpassing the teat end. Similar results after intracisternal challenge were obtained by Brooks and Barnum (1984). When experimental challenge consisted of exposure of the teat orifice to *Staph. aureus*, no protective effect of corynebacteria was found (Brooks and Barnum, 1984). This underscores the importance of the teat end in (non-)occurrence of new infections. Results from Lam et al. (1997) are ambiguous with respect to the effect of infection with corynebacteria on subsequent infection with *Staph. aureus*. Our study on naturally occurring infections showed that the role of minor pathogens differs with minor pathogen species, and with herd-, cow-, and quarter-level factors, including teat-end callosity. Together with the many different study types, challenge methods and definitions of infection that have been used, this may explain why conflicting results have emerged from numerous studies on the role of minor pathogens. Knowing that postmilking teat disinfection (PMTD) affects the prevalence of minor pathogens (Lam et al., 1997), it is interesting that herds A and B did not use PMTD throughout the entire study or parts of the study, as opposed to herd C. This difference in management may be associated with the

difference in the significance of corynebacteria as risk factor for IMI. Unfortunately, with only three herds enrolled in this study, a role of management factors cannot be proven.

As discussed earlier, multiple infections within a cow occur at a higher rate than would be expected based on independence of quarters, and this result may be due to increased susceptibility of certain cows or to within-cow transmission of pathogens (Barkema et al., 1997). In our study, exposure to other quarters infected with *Strep. uberis* within a cow was associated with increased rate of *Strep. uberis* infection. Exposure to other quarters infected with *Staph. aureus* within a cow was associated with increased rate of *Staph. aureus* infection. Because clustering at cow level and several cow and quarter-level risk factors associated with susceptibility were accounted for in the model, this suggests that within-cow transmission does occur for both pathogens.

Bulk milk SCC is decreasing in several countries, but the decline in BMSCC has not been accompanied by a decrease in incidence of clinical mastitis (Milteneburg et al., 1996; Peeler et al., 2000). On the contrary, studies on clinical mastitis in herds with low BMSCC have led to the suggestion that low SCC may be associated with increased risk or severity of mastitis (Barkema et al., 1998; Suriyasathaporn et al., 2000a). In our study on naturally occurring infections, low quarter SCC was not associated with an increased rate of subsequent infection. This is in contrast to results obtained by others. In an experimental study, low prechallenge SCC at quarter level was associated with an increased risk of *Staph. aureus* mastitis (Schukken et al., 1999). Quarters were challenged by intracisternal infusion of *Staph. aureus*, thus eliminating the role of the first line of defense that is important in natural infections. This may explain the difference in results between the experimental study and our field study. In a case-control study on low SCC as risk factor for mastitis, IMI with clinical signs was associated with lower preinfection SCC than IMI without clinical signs (Suriyasathaporn et al., 2000a). The case-control study was carried out as a field study in a low bulk milk SCC herd. The majority of clinical mastitis cases yielded no growth or growth of *E. coli* while less than 15% of clinical cases yield *Strep. uberis* or *Staph. aureus*. The disagreement between studies with respect to effect of low SCC on subsequent mastitis is probably the result of differences in study design, herd type, pathogen, and definitions of mastitis. It is a reminder that conclusions based on one pathogen in one type of study or one herd cannot automatically be generalized to other pathogens, other categories of animals at risk, or other types of herds.

Missing values for SCC were not missing at random. Therefore, they were coded as specific categories. Values were mostly missing when the preceding sample was a farmer-collected sample. Level 6 generally indicated that the preceding sample was taken at calving (data not shown). The significance of pSCC level 6 for *Staph. aureus* could be interpreted as evidence that the rate of new infection with *Staph. aureus* was high when animals were introduced to the milking parlor after calving, although early lactation (1 to 60 DIM) was not associated with an increased rate of infection. The difference between the presence of infection at calving and onset of infection in early lactation is relevant because management strategies should be targeted at the nonlactating or the lactating period, depending on the time of occurrence of new infections. A larger dataset would be needed to discriminate between the two scenarios. In addition, strain typing studies could be used to differentiate between transmission in the milking parlor and infection from other sources (Zadoks et al., 2000).

The system that was used to classify teat-end callosity was recently developed by Neijenhuis et al. (2000). The study reported here is the first to examine the role of teat-end callosity as a risk factor for IMI, including subclinical mastitis. An increased rate of *Staph. aureus* infection was observed in quarters with extreme thickness of teat-end callus and in quarters with rough teat ends that were infected with corynebacteria. An effect of TECR or TECT on the rate of *Strep. uberis* infection was not found. The mechanism through which teat-end callosity and *Staph. aureus* IMI are associated is unknown. Poor milking machine function could lead to teat-end callosity and to increased risk of *Staph. aureus* IMI. In that case, both factors are the result of a common cause, even though teat-end callosity preceded IMI in time. Alternatively, rough and extremely callused teat ends themselves could harbor *Staph. aureus*. Further research is needed to explain the difference between effects of teat-end callosity on rate of *Staph. aureus* IMI and *Strep. uberis* IMI, and to unravel the biological relation between teat-end callosity and infections with minor and major pathogens.

## CONCLUSIONS

The rate of infection with *Strep. uberis* and *Staph. aureus* was lower in first- and second-parity cows than in older cows. There was no association of infection rate with BCS, or change in BCS, while association of lactation stage with infection rate differed with herd and with pathogen. BHV4-infection may play a role in susceptibility to mastitis, especially for *Staph. aureus*. Quarters that recovered from infection with *Strep. ub-*

*eris* or *Staph. aureus* showed an increased rate of new infection with either pathogen. Quarters that were exposed to *Strep. uberis* or *Staph. aureus* within the same cow show an increased rate of new infection with the homologous pathogen. There was no effect of minor pathogens on subsequent infection with *Strep. uberis*, while the effect on rate of *Staph. aureus* infection depended on minor pathogen species, herd, stage of lactation, and teat-end callosity. Low-quarter SCC was not associated with increased rate of IMI with *Strep. uberis* or *Staph. aureus*. Teat-end roughness and extreme teat-end callosity increased the rate of *Staph. aureus* mastitis but not *Strep. uberis* mastitis.

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